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Immunoprophylactic studies on cell wall associated proteins of *Mycobacterium tuberculosis* $H_{37}Ra$

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Abstract. The cell wall protein peptidoglycan complex (CW-PPC) of *Mycobacterium tuberculosis* $H_{37}Ra$ was isolated through sequential extraction of lipids, carbohydrates and soluble proteins. CW-PPC emulsified in FIA was found to induce significant protection in mice against challenge with LD_{50} dose of *M. tuberculosis* $H_{37}Rv$. To identify the immunoprotective components of CW-PPC, the proteins in avid association with peptidogican were dissociated by chemical treatment with trifluoromethanesulthonic acid (CF_3CO_3H): anisole (2:1). Immunoreactivity of total (CW-Pr) as well as its component proteins i.e., 71, 60 and 45 kDa proteins of cell wall was studied in animals immunized with CW-Pr-FIA. The 71 kDa protein was found to be most immunoreactive giving higher T-cell sensitization and humoral responses. Further, immunization of mice with 71 kDa-FIA demonstrated enhanced T- and B-cell responses. Mice immunized with 71 kDa-FIA gave significantly higher protection ($P \leq 0{\cdot}05$) against intravenous challenge with LD_{50} dose of *M. tuberculosis* $H_{37}Rv$, than BCG immunized animals. The results indicate the potential of 71 kDa cell wall protein as a suitable candidate for Cthe subunit vaccine.

Keywords. Tuberculosis; cell wall; protein antigens; vaccine.

1. Introduction

Tuberculosis caused by *Mycobacterium tuberculosis* continues to be a major health problem, especially in the developing countries. The efficacy of presently available antituberculous vaccine BCG is highly variable, with an average reduced risk of only 50% against tuberculosis (Colditz *et al* 1994). Hence, studies are in progress to identify immunoprotective agents as alternatives to BCG.

Several studies indicate that mycobacterial cell walls are likely to contain T-cell antigens capable of eliciting protective immune responses (Ribi *et al* 1982; Melancon-Kaplan *et al* 1988; Barnes *et al* 1989). Presently, much attention has been focussed on the immunological reactivity of non-extractable, muramidase resistant polypeptides associated with mycobacterial cell wall peptidoglycan (Hirschfield *et al* 1990). These proteins are known to elicit delayed type hypersensitivity response in sensitized humans and animals by activating appropriate T-lymphocytes (Anacker *et al* 1969; Mehra *et al* 1989). Therefore, cell wall peptidoglycan associated proteins are suggested to be important immunodominant antigens of mycobacteria. However, no study has been carried out on the immunoreactivity of these proteins. In the present study, we have isolated cell wall protein peptideglycan complex (CW-PPC) and dissociated its proteins by chemical treatment with

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trifluoromethanesuiphonic acid in order to investigate its immunoprophylactic properties.

2. Materials and methods

2.1 *Bacterial cultures*

M. tuberculosis $H_{37}Ra$, *M. tuberculosis* $H_{37}Rv$ and *M. bovis* BCG were obtained from the National Collection of Type Cultures (NCTC), London, and maintained on either Lowenstein-Jensen's medium or on modified Youman's medium.

2.2 *Animals*

Mice of NMRI strain, of either sex (4-5 weeks old) were obtained from the Central Research Institute, Kasauli. The mice were fed on a standard pellet diet and given water *ad libitum.*

2.3 *Isolation of antigens*

CW-PPC was isolated from M. *tuberculosis* $H_{37}Ra$ cells as described previously (Chugh and Khuller 1993).

For the release of CW-PPC bound polypeptides, method of Hirschfield *et al* (1990) was followed with slight modifications. Briefly, disrupted cells were subjected to sodium dodecyl sulphate (SDS) extraction to remove SDS soluble proteins. Release of peptidoglycan bound proteins was facilitated by, CF_3SO_3H: anisole (2:1) treatment followed by diethyl ether and pyridine extraction. The aqueous phase recovered was dialyzed and insoluble cell wall protein (CW-Pr) was separated by centrifugation and solubilized in sample buffer. Preparative SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laeminli (1970). The individual proteins were eluted from the gel by cutting the corresponding bands and homogenizing with DDW followed by centrifugation. The supernatant containing pure protien was then dialyzed against phosphate buffered saline (PBS, pH 7·3).

2.4 *Immunization*

Animals (in group of 20 each) were immunized subcutaneously on day 0 and 7 followed by an intramuscular injection on day 14 in following groups.

The first group received CW-Pr (60 µg per animal) divided into three equal doses of 20 µg each, emulsified in 20 µl of Fruends incomplete adjuvant (FIA). Similarly, the second group received 71 kDa (60 µg per animal) in three equal doses in FIA. The third group received normal saline in FIA which served as control.

2.5 *Immune responses*

At weekly intervals, 4-5 animals were exsanguinated and their spleens were removed aseptically. The sera were stored at — 20°C until further use.

2.6 *Lymphocytes cultures*

T-cell transformation assay was performed as described by Pal and Horwitz (1992). Briefly, 10 µl of lymphocytes (2×10^6/ml) were dispensed in each well of flat bottomed 96-well tissue culture plate (Corning, New York) and incubated at 37°C in 5% CO_2 with optimal antigen concentration. After 4 days, cultures were pulsed with [^{3}H] thymidine at a concentration of 0·5 µCi/well (Bhabha Atomic Research Centre, Bombay) for 18 h and incorporation of [^{3}H] thymidine into proliferating cells was measured as cpm by liquid scintillation counter (LKB Wallac, 1214, Rack beta ISC). PHA (1 µg/well) was used as positive control. The stimulation index was calculated as

$$\text{SI} = \frac{\text{cpm of antigen stimulated cells}}{\text{cpm of unstimulated cells}}.$$

2.7 *Humoral immune responses*

Enzyme linked immunosorbant assay (ELISA) was used to detect antibodies against individual antigens. The optimum concentration of antigens (71, 60, 45 kDa and CW-Pr; 10 µg/ml) were used for coating the microtitration plates. Goat anti-mouse lgM peroxidase conjugate (Sigma) diluted 1:2000 (v/v) was used as enzyme and *o*-phenyl diamine (34 mg in 100 ml of 0·15 M citrate–phosphate buffer; pH 5·0) as substrate. Absorbance of test plates was read at 492 nm using ELISA plate reader (Diamedix, Microassay Reader).

2.8 *Protection studies*

To assess the protection induced on immunization with CW-PPC-FIA (150 µg per animal), CW-PPC-FIA and 71 kDa-FIA animals (in group of 20 each) were immunized in same schedule as described previously. At three weeks post-immunization (p.im.), the animals were challenged intravenously with sublethal dose (LD_{50}: 3 x 10^7 organisms/mice) of M. *tuberculosis* H_{37}Rv. The BCG immunized group received 1 x 10^6 organisms per animal intravenously in single dose. The protection induced was evaluated by observing the survival rates as described by Pancholi *et al* (1989).

2.9 *Statistical analysis*

The data of immune responses was evaluated using Student's '*T*' test. The protection data was analysed using x^2 test.

3. Results

3.1 *Immunoprotection of mycobacterium CW-PPC*

The protection afforded by mycobacterial CW-PPC complexed in FIA against challenge with LD_{50} dose of M. *tuberculosis* H_{37}Rv was determined in comparison to control. The CW-PPC immunized group demonstrated 90% survival which was

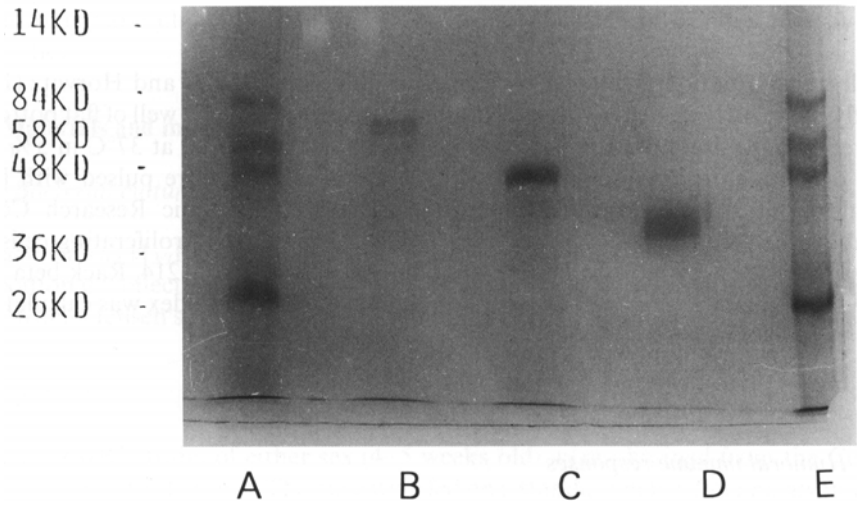

Figure 1. SDS-FAGE of isolated cell wall proteins compared to molecular weight markers. Triose isomerase (26 kDa), LDH (36 kDa), Furnarase (48 kDa), pyruvate kinase (58 kDa), Fructose-6-phosphate kinase (84 kDa) and ß-galactosidase (114 k Da) obtained from sigma served as molecular weight markers. Lane A and E, molecular weight markers; lane B, 71 kDa; lane C, 60 kDa; lane D, 45 kDa.

significantly higher ($P \leq 0.01$) than the control group (60%). Further to identify the immunoprotective component of CW-PPC, its associated proteins were released to carry out detailed studies on their immunoreactivity.

3.2 *Isolation of proteins associated with CW-PPC*

The chemical treatment of SDS-extracted cell wall with CF_3SO_3H resulted in non-dialyzable, insoluble cell wall protein (CW-Pr). The SDS-PAGE of CW-Pr showed three bands which when compared with standard molecular weight markers were found to be of 71, 60 and 45 kDa (figure 1).

3.3 *Immunoreactivity of cell wall associated proteins*

Both cellular (T-cell activation) and humoral (ELISA) immune responses were studied at weekly intervals p.im. against total and individual isolated proteins i.e., CW-Pr, 71, 60 and 45 kDa in animals immunized with CW-Pr-FIA. Maximum T-cell sensitization was observed to 71 kDa protein at second ($SI = 6.91 \pm 0.23$, $P \leq 0.001$) and third ($SI = 8.39 \pm 0.43$, $P \leq 0.001$) week p.im. which was significantly higher as compared to CW-Pr, 60 and 45 kDa proteins (figure 2a).

CW-Pr emulsified in FIA also induced significant humoral responses to all the antigens. Maximum antibody production was seen at second week p.im. where absorbance values at 492 nm were found to be 1.126 ± 0.095, 1.048 ± 0.073, 1.053 ± 0.021 and 0.685 ± 0.035 in response to stimulation with 71, 60, 45 kDa and CW-Pr respectively. All these values were significantly higher ($P \leq 0.001$) as compared to corresponding control values which were 0408 ± 0.03, 0439 ± 0.03, 0.352 ± 0.02 and 0.358 ± 0.073 respectively.

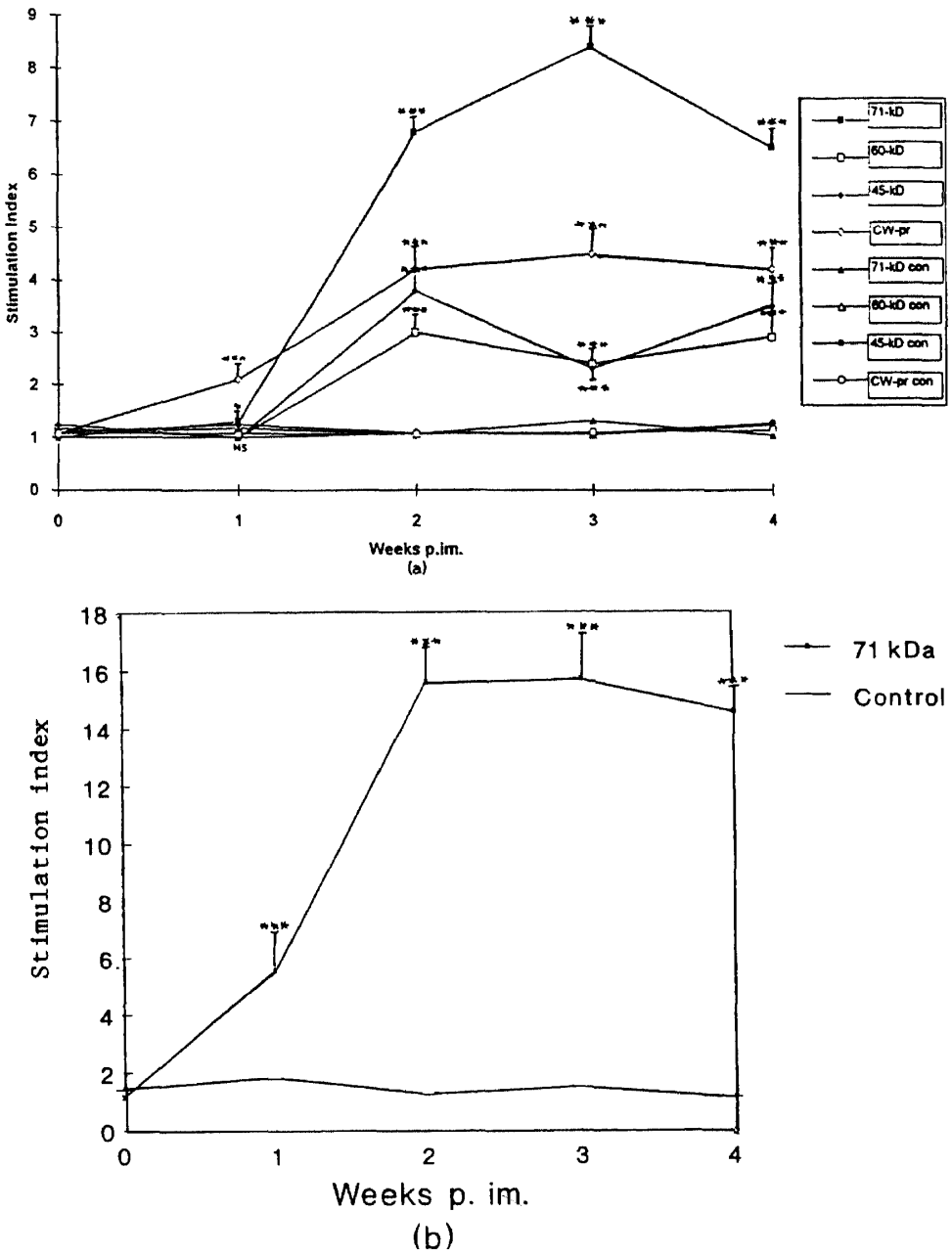

Figure 2. Comparative study of proliferative responses elicited to CW-Pr, 71, 60 and 45 kDa in animals immunized with CW-Pr-FIA (a) and proliferative responses elicited to 71 kDa protein in animals immunized with 71 kDa-FIA (b). SIB (stimulation index) is mean [^{3}H] incorporation in lymphocytes incubated with antigen/mean [^{3}H]incorporation in lymphocytes incubated without antigen. All values are mean of 4–5 animals. ***$P \leq 0{\cdot}001$; **$P \leq 0{\cdot}01$ with respect to control.

These results suggest 71 kDa to be the most reactive protein out of the isolated proteins i.e., 71, 60 and 45 kDa. Hence, animals were immunized with 71 kDa-FIA and their immune responses were studied.

3.4 *Immune responses to 71 kDa protein*

Both T-and B-cell immune responses in 71 kDa immunized animals were examined as described earlier. The T-cell proliferative response of the lymphocytes obtained from 71 kDa immunized animals was maximum at second and third week p.im. (figure 2b). This response was significantly higher from those obtained in CW-Pr immunized animals (figure 2a). Significant antibody production was observed by 71 kDa protein as compared to control at all weeks p.im. Maximum response was seen at second week p.im. (1·614 ± 0·19) which was singnificantly higher ($P \leq 0{\cdot}001$) as compared to control (0·459± 0.084).

3.5 *Protective efficacy of cell wall proteins*

At the peak of immune responses, immunized as well as control mice were challenged with LD_{50} dose of *M. tuberculosis* $H_{37}Rv$ (3×10^7 CFU/mouse). Immunization with CW-Pr and 71 kDa protein emulsified in FIA resulted in increased survival of immunized mice. At 30 days p.im., net protection in 71 kDa immunized animals (92%) was better as compared to CW-Pr (84%) and was significantly higher ($P \leq 0{\cdot}05$) than BCG immunized (50%) calculated on the basis of survival rates (figure 3).

4. Discussion

Protective immunity to tuberculosis is thought to be mainly cell mediated. T-cells secrete various macrophage and T-cell activating interleukins, which are the mediators of acquired resistance against tuberculosis in human and murine systems (Boom *et al* 1987; Orme 1987; Orme and Collins 1984). Recent studies, indicating the ability of mycobacterial cell wall associated proteins to activate T-lymphocytes from tuberculous patients, make them a suitable candidate for antituberculous vaccine (Barnes *et al* 1989).

In the preliminary studies, significant protection (80%) was observed by immunization of animals with CW-PPC as seen by increased survival rates (90%) in immunized animals as compared to controls (60%). These findings are in accordance with previous reports on mycobacterial cell wall affording protection against tuberculosis infection (Anacker *et al* 1969; Ribi *et al* 1982; Barnes *et al* 1989). Further, the reactivity of cell wall peptidoglycan has been reported to be due to some protein constituents as its imrnunoreactivity is lost after proteinase or pronase K digestion (Melancon-Kaplan *et al* 1988; Misaki *et al* 1966). Therefore, the proteins associated with peptidoglycan were dissociated to study their immune responses.

Study of the immune responses to individual protein antigens i.e., 71, 60, 45 kDa and CW-Pr in mice immunized with CW-Pr showed significant T- and B-cell immunoreactivity (figure 2a). As far as B-cell reactivity was concerned, there was significant antibody response to isolated antigens with maximum to 71 kDa. Similarly, the cell

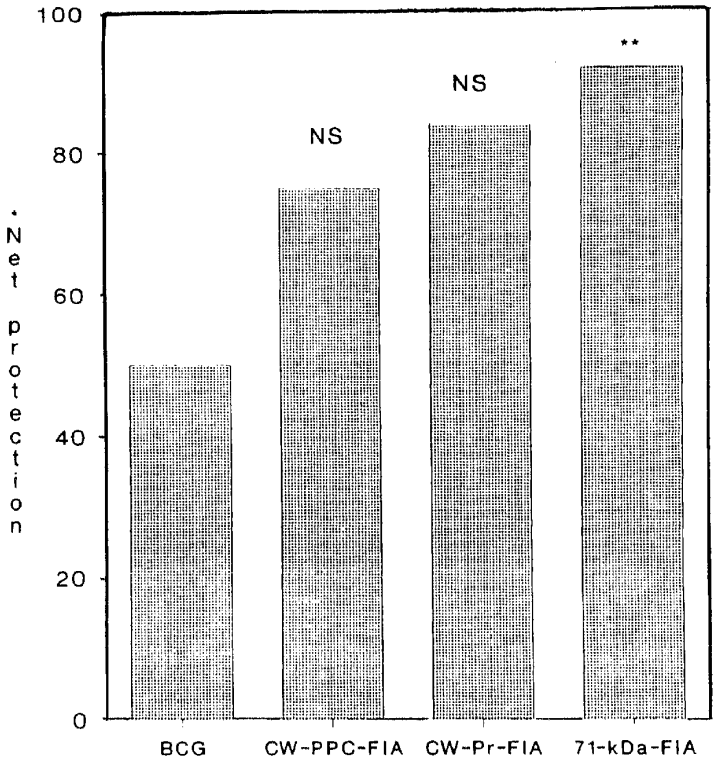

Figure 3. Comparison of per cent protection afforded by immunization with CW-PPC-FIA, CW-Pr-FIA, 71 kDa-FIA and BCG against challenge with LD_{50} dose of M. *tuberculosis* H_{37} Rv. *Per cent protection was calculated by assuming the mortality in control animals 100% at the end of 30 days of infection. **F < 0·05, NS non significant calculated with respect to BCG immunized.

mediated immune responses to 71 kDa protein were also found to be highest as indicated by maximum T-cell proliferation. These results are similar to our earlier findings, in which the 71 kDa cell wall protein showed maximum immunoreactivity with tuberculous and tuberculin positive healthy individuals as compared to other cell wall antigens (Dhiman N, Verma I and Khuller G K, unpublished results).

As 71 kDa antigen was found to be most immunoreactive amongst the antigens investigated, detailed immunoprophylactic studies were carried out with this antigen using FIA as adjuvant. Clear evidence of cell mediated reactivity of 71 kDa protein was obtained as indicated by significantly higher T-cell activation in immunized animals which was better than CW-Pr-FIA immunized animals. This might be due to the presence of 45 and 60 kDa proteins in CW-Pr which have lower immunoreactivity and might be influencing the immunoreactivity of 71 kDa protein. Another reason could be that immunization with CW-Pr does not provide the effective immunization dose of 71 kDa to the animals. Further, significantly higher levels of IL-2 and IFN-r release was observed in 71 kDa immunized mice (data not shown). These results suggest the activation of TH_1 cell type, which are mediators of cellular immunity and are important for the induction of protective immunity (Flynn *et al* 1993; Cooper *et al* 1994).

The immune responses elicited by 71 kDa were further found to be protective as immunization with this antigen provided significant protection against challenge with

LD_{50} dose of *M. tuberculosis* $H_{37}Rv$. The protective efficacy of 71 kDa (92%) was found to be slightly better than CW-Pr (84%) and significantly higher ($P \leq 0{\cdot}05$) than BCG (50%), thus indicating better potential of 71 kDa cell wall antigen as compared to standard BCG vaccine.

In conclusion, in this study 71 kDa protein of mycobacterial cell wall has been identified as the major immunoprotective antigen responsible for imparting protection against tuberculosis and is a potent candidate for future subunit vaccine.

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References

Anacker R L, Barclay W R, Brehmer W, Goodie C, List F H, Ribi E and Tarmina D F 1969 Effectiveness of cell walls of *Mycobacterium boris* strains of BCG administered by various routes and in different adjuvants in protecting mice against air borne infection of *M. tuberculosis* strain H_{37} Rv; *Am. Resp. Resp.Dis.* **99** 242-248

Barnes P F, Mehra V, Hirschfield G R, Fong S, Abou-Zeid C, Rook G A W, Hunter S W, Brennan P J and Modlin R L 1989 Characterization of T-cell antigens associated with cell wall protein peptidoglycan complex of *Mycobacterium tuberculosis; J. Immunol.* **143** 2656-2662

Boom M H, Husson R N, Young R A, David J R and Piessens W F 1987 *In vivo* and in *vitro* characterization of murine T-cell clones reactive to *Mycobacterium tuberculosis; Infect. Immun.* **55** 2223-2229

Chugh I B and Khuller G K 1993 Immunoprotective behaviour of liposome entrapped cell wall subunit of *Mycobacterium tuberculosis* against experimental tuberculous infection in mice; *Eur. Resp. J.* **6** 811-815

Colditz G A, Brewer T F, Berkley C S, Wilson M E, Burdick E, Fineberg H V and Mosteller F 1994 Efficacy of BCG vaccine in the prevention of tuberculosis; *J. Am. Med. Assoc.* **271** 698-702

Cooper A M, Dalton D K, Stewart T A, Griffin J F, Russel D G and Orme I M 1994 Disseminated tuberculosis in Interfernon-gamma gene disrupted mice; *J. Exp. Med.* **178** 2243-2247

Flynn J L, Chan J, Triebold K J, Dalton D K, Stewart T A and Bloom B R 1993 An essential role of Interferon-gamma in resistance to *Mycobacterium tuberculosis* infection; *J. Exp. Med.* **178** 2249-2254

Hirschfield G R, McNeil M and Brennan P J 1990 Peptidoglycan associated polypeptides of *Mycobacterium tuberculosis; J. Bacterial.* **172** 1005-1013

Laemmli U K 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T_4; *Nature* (*London*) **227** 680-685

Mehra V, Bloom B R, Torigian V K, Mandich D, Reichet M, Young S M M, Salgame P, Convit J, Hunter S W, McNeil M, Brennan P J, Rea T H and Modlin R L 1989 Characterization of *Mycobacterium leprae* cell wall associated proteins with the use of T-lymphocyte clones; *J. Immunol.* **142** 2873-2878

Melancon-Kaplan J, Hunter S W, McNeil M, Stewart C, Modlin R L Rea T H, Convit J, Salgame P, Mehra V, Bloom B R and Brennan P J 1988 Immunological significance of *Mycobacterium leprae* cell walls; *Proc. Natl. Acad. Sri. USA* **855** 1917– 1921

Misaki A, Yutawa S, Tsuchiya K and Yamasaki T 1966 Studies on the cell walls of mycobacteria. 1. Chemical and biological properties of the cell walls and the mucopeptide BCG; *J. Biochem.* **59** 388-396

Orme I M 1987 The kinetics of emergence and loss of mediater T lymphocytes acquired in response to infection with *Mycobacterium tuberculosis; J. Immunol.* **138** 293-298

Orme I M and Collins F M 1984 Adoptive protection of *Mycobacterium tuberculosis* infected lung: dissociation between cells that passively transfer protective immunity and those that transfer delayed type hypersensitivity to tuberculin; *Cell. Irnmunol.* **84** 113-120

Pal P G and Horwitz M A 1992 Immunization with extracellular protein of *Mycobacterium tuberculosis* induces cell mediated immune responses and substantial protective immunity in guinea pig model of pulmonary tuberculosis; *Infect. Immun.* **60** 4781-4792

Pancholi P, Vinayak V K and Khuller G K 1989 Immunogeriicity of ribonucleic acid protein fraction of *Mycobacterium tuberculosis* encapsulated in liposomes; *J. Med. Micorbial.* **29** 131—138

Ribi E, Granger D L, Milner K C, Yamamoto K, Strain S M, Parker R, Smith R W, Brehmer W and Azuma 11982 Induction of resistance to tuberculosis in mice with defined components of mycobacteria and with some unrelated materials; *Immunology* **46** 297-301